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VIRUS INDEXATION OF *IN VITRO* REGENERATED SUGARCANE PLANTS

SHAGUFTA NAZ[1*], FAYYAZ AHMAD SIDDIQUI[2], AAMIR ALI[3] AND JAVED IQBAL[4]

[1]Department of Botany, Lahore College for Women University, Lahore, Pakistan
[2]AgriBiotech, 14-Km Multan Road, Lahore, Pakistan
[3]Department of Biological Sciences, University of Sargodha, Sargodha, Pakistan
[4]School of Biological Sciences, University of the Punjab, Quaid-e-Azam Campus, Lahore, 54590, Pakistan.

Abstract

The present study reports the SCMV indexation of *In vitro* regenerated plants by infectivity assay, serology test and by transmission electron microscopy. The plants were developed by apical meristem, organogenesis and embryogenesis methods. For infectivity test sap of *In vitro* regenerated plants was inoculated on different cultivars of Sorghum. Out of 166 plants only 44 were SCMV+tive. These plants were further analyzed by using precipitin test. These plants also showed negative result for virus. A total of 17 randomly selected plants i.e., 2 from control, 5 each from apical meristem, organogenesis and somatic embryogenesis were processed for electron microscopy. The control plants showed virus particles. No virus particles were detected from plants derived from apical meristem. Two plants obtained from organogenesis and three from embryogenesis were identified with virus particles. All the SCMV indexed plants were grown in green house and monitored for mosaic symptoms at weekly intervals.

Introduction

A significant proportion of the total world crop production is lost each year because of various pathogens like viruses, bacteria, fungi and nematodes (Ahmad *et al.*, 2007). Diseases caused by fungi and bacteria have been successfully controlled chemotherapeutically. Unlike fungi and bacteria there is no chemical or physical treatment to eradicate effectively viruses from infected plants. This is mostly due to the fact that viruses do not have independent metabolism. They mobilize the metabolic machinery of the infected plant so that they multiply at the expense of the host metabolism. These events in the virus infected plant lead to depletion of or accumulation of or appearance of new compounds and lead to induce biotic stress to the host. Chemotherapeutic interference of viral replication and synthesis could not be done without adverse effect on the host nucleic acid and protein synthesis mechanism (Rao *et al.*, 2001).

Virus infected plants either deteriorate quality or reduce the yield to a significant level (Rassaby, 2003; Wang & Hu. 1980; Kartha, 1986; Sreenivalsu *et al*., 1989). It has been reported that replacement of virus infected stock with the healthy stock (virus free) has shown up to 300% yield increase (Murashige, 1980;Schenck & Lehrer 2000). It is an established fact that vegetatively propagated plants once systematically infected with a virus, the pathogen passes from one vegetative generation to the next. The entire population of a given clonal variety plant may over a year be infected with the same pathogen (Schenck & Lehrer, 2000).

*Corresponding author E-mail: drsnaz31@hotmail.com

Sugarcane is one of the important cash crops of Pakistan. It contains a major source of edible sugars. Many by-products are also produced from sugarcane (Raja, 2006). Wide use of sugar and its relevant products have created a challenging situation for sugarcane researchers and growers. In spite of extensive research the average yield of sugarcane in Pakistan is very low as compared to other cane producing countries of the world (Imam, 2001). There are too many factors responsible for low yield but most striking one is its extreme susceptibility to pathogens, especially viruses. Sugarcane alone is infected by five major virus diseases, mosaic, streak, sereh, Fiji and ratoon stunting (Skykhuis, 1976). Sugarcane mosaic virus (SCMV) is widely spread and almost all the cultivars grown in the continent are infected with the virus. A significant part of yield (39%-46%) is lost every year due to SCMV harboring (Singh, 1971). Mandahar (1987) reported that the disease occurs in all the sugarcane growing parts of the world to an extent that it is almost difficult to find a single healthy plant in the field.

Sugarcane is vegetatively propagated plant hence propagation of infected plant transmits SCMV generation after generation. Lack of flowering potential, virus resistance and efficient multiplication procedures have long been serious problem in sugarcane breeding. Significantly the earliest awareness of the potential for sugarcane improvement appeared when tissue culturist was closely associated with plant breeders and pathologist. Initiation of sugarcane tissue culture was first reported by Heinz & Mee (1969) An intensive work for sugarcane improvement by using this technique has been initiated by Liu (1983) by getting callus induction and subsequent regeneration by using immature inflorescence, apical meristem, young leaves and pith parenchyma. With the passage of time, more emphasis was focused on the elimination of viruses by using apical meristem. (Mori, 1971; Leu, 1972).

Since the invent of *In vitro* techniques, a lot of interest has been generated in the recent year for the rapid multiplication of virus free sugarcane through apical meristem, (Ali *et al*., 2007), somatic embryogenesis (Naz *et al*., 2008) and callus cultures (Ali *et al*., 2008). Parmessur (2002) reported the use of tissue culture as a means to eliminate both SCYLV and SCYP from exotic varieties undergoing quarantine in Mauritius. Yellow leaf syndrome (YLS) is a recently reported disease of sugarcane, characterized by yellowing of the leaves. Two pathogens: a virus**,** Sugarcane yellow leaf virus (SCYLV); and a phytoplasma, sugarcane yellows phytoplasma (SCYP) are associated with the disease.

Successful elimination of sugarcane mosaic virus by tissue culture methods has been reported by many workers (Dean, 1982; Irvine & Benda, 1987; Peros *et al*., 1990). Much of the interest was focused on explant source, effect of media composition on virus eradication and frequency of regeneration and serological technique for detection of SCMV. The evidences also indicated that SCMV positive tissue in cultures of certain incubation periods produce negative symptoms. The intensive bioassays regenerated plants revealed the substantial number of symptom-less stock successfully rose through *In vitro* techniques.

Therefore, in view of the above, the present invention is directed towards the SCMV indexation of *In vitro* regenerated plants either developed from apical meristem, organogenesis and somatic embryogenesis by infectivity assay, serology test and by transmission electron microscopy.

Materials and Methods

The off shoots of *Saccharum officinarum* L. cv Col. 54 were obtained from Ayub Agriculture research station, Faisalabad. Shoot apical meristem, spindle leaves and pith parenchyma in different sizes were used as explants for micropropagation, callus

induction and for somatic embryogenesis. Sugarcane explants raised by these methods were used for virus assay. Six weeks old plants from green house were randomly selected and used for further study.

To determine the failure of sugarcane mosaic virus (SCMV) to survive in cultured sugarcane tissue, three commonly used bioassay methods based on infectivity assay, serology and electron microscopy were used.

Infectivity assay: Infectivity test or sap transmission of virus is one hundred time more sensitive than serological tests and electron microscopy (Holling & Stone 1960; Baker & Kinnman, 1973; Wang & Hu, 1980). To determine the presence of SCMV, the *In vitro* regenerated sugarcane plants raised from meristem, callus or embryo cultures and growing in Green House were used. After sixth week of establishment of plants, a part of the youngest visible leaf from each source were triturated in a few drops of distilled water and ground into sap. Leaves of one-week-old indicator plants of different cultivars of Sorghum (JS-1, Bagdar, PU-7 and Red Janpur L.) propagated in green houses were slightly dusted with 600-grade carborundum. Sorghum plants were chosen for assay host because of its extreme susceptibility to SCMV and intense symptoms (Dean, 1982). The sap (inoculum) was mechanically rubbed hard enough to infect the surface cell of the leaf. After five minutes the inoculated leaves were gently washed with water to remove the residual inoculum. Inoculated plants were maintained under the glass house for several weeks for symptom development. Symptoms on the sorghum plants were noted at weekly intervals for four weeks. Sugarcane plant indexing negative were further assayed for virus presence by serological method and electron microscopy.

Serology: Serology assay is based on the specificity between antigen and antibodies resulting in a visible or measurable reaction product. Among the serological reaction, precipitation or precipitin reaction were used in the study. Precipitin test is relatively sensitive and has been extensively used for virus indexation (Koike & Gillespie, 1976; Kartha, 1986; Mandahar, 1987).

To perform the reaction, a series of test tubes (7 mm diameter) were kept in water bath adjusted at 50°C. One ml of an anti-serum (SCMV-PVAS 186, American type culture collection) at fixed dilution (1/10 ml: I ml anti-serum dissolved in 10 ml of sterile distilled water) were added to each test tube and then 1ml of antigen (APP) at various dilution (from 0.01to 0.5) prepared serially by two fold dilution, were added to each test tube. The contents were thoroughly mixed and carefully observed for the first appearance of precipitation. The precipitation was the indication of virus detection (+ve) and clear solution virus detection (–ve).

Electron microscopy: For electron microscopy, 12 plants were selected randomly for virus detection. The crude extract of each plant was mounted on mesh carbon coated copper grids stained with 2% PTA (Phosphotungstic acid, pH 6.8. Grids were examined under TEOL 100CX-11 electron microscope at 80 KV)

Results

Sugarcane plants raised through apical meristem, organogenesis *via* callus and embryogenesis were used for virus assay. Six weeks old plants from Green House were randomly selected for virus indexation. Virus Indexation was carried: (i) by infectivity assay (ii) serology tests and (iii) by transmission electron microscopy.

About 75 plants were raised from original SCMV infected stock. All plants raised from the stock were treated as control. Randomly selected 216 plants, of control (50), raised through shoot apical meristem (46), organogenesis (54) and embryogenesis (66) were analyzed for SCMV disease.

Infectivity test: To determine the SCMV-symptoms, sap of severely infected sugarcane plants (control) was to inoculate mechanically on the host plants i.e., *Sorghum bicolor* at 3 leaf stages. All the inoculated plants developed severe mosaic symptoms. The first post inoculation symptom on sorghum consisted of small chlorotic spots appearing at the terminal whorl of youngest leaf. The number of spots increased as the disease progressed; spots became linearly elongated as the leaves increased in size resulting in chlorotic stripes (Fig. 1b). All these symptoms closely correlate with SCMV symptoms in sugarcane (Fig. 1a)

Out of total 216 plants, 166 were derived through *In vitro* cultures. Among these plants 46 were from apical meristem, 54 from organogenesis and 66 from embryogenesis origin. All plants were tested for the presence of SCMV on sorghum. A total number of 114 of these plants did not produce any of the infection symptoms in sorghum, while 102 plants produced viral symptoms. From shoot apical meristems 43, organogenesis 33 and embryogenesis 38 healthy plants were obtained. Only 3 plants rose from apical shoot meristems, 21 from organogenesis and 28 from embryogenesis were found infected (Fig. 1c). All the control plants exhibited clear SCMV symptoms. In terms of percentage, 93.70% virus-free plants were obtained from meristem, 61.11% from organogenesis and 57.57% from embryos (Table 1)

Serology: All the plants used for infectivity tests were further analyzed by using precipitin test to determine the SMCV positive. All samples tested from control plants produced clear precipitation as a result of antigen anti body reaction and hence were SCMV positive. Of the *In vitro* grown plants, indexed negative with infectivity tests also showed negative results in precipitin test. Furthermore regenerated plants positive in infectivity test were also positive for SCMV in precipitation test (Table 1).

Electron microscopy: A total of 17 randomly selected plants i.e. 2 from control, 5 each from apical meristem, organogenesis and embryogenesis were processed for electron microscopy. The control plants showed virus particles. Of the 5 plants obtained through apical meristem, all plants were without virus particles and showed 100% SCMV virus free plants. In organogenesis, 3 were without virus and 2 were identified with virus particles. Only 2 from embryogenesis were virus free and 3 had virus particles. Higher yield of virus free plants was obtained from the stock raised from apical meristem (100%), while plants derived from embryogenesis exhibited lowest yield of virus free plants (20 %) (Table 2).

All the indexed plants (Fig. 1d, e) were grown in green house and monitored for mosaic symptoms at weekly basis.

Effect of meristem size on virus elimination: The size of meristem played a pivotal role in elimination of virus in micro-propagated plants. In one of the experiments, different sizes (0.2-5.0 mm) of apical meristems were cultured. Plants regenerated from meristems of size 0.2-3.0 mm were all free of SCMV symptoms, while one plant derived from 4.0 mm and two from 5.0 mm size meristems showed SCMV symptoms. This indicated that the size of the meristem was also a determining factor in elimination of the virus. The larger apices were more prone to retaining virus compared to smaller one.

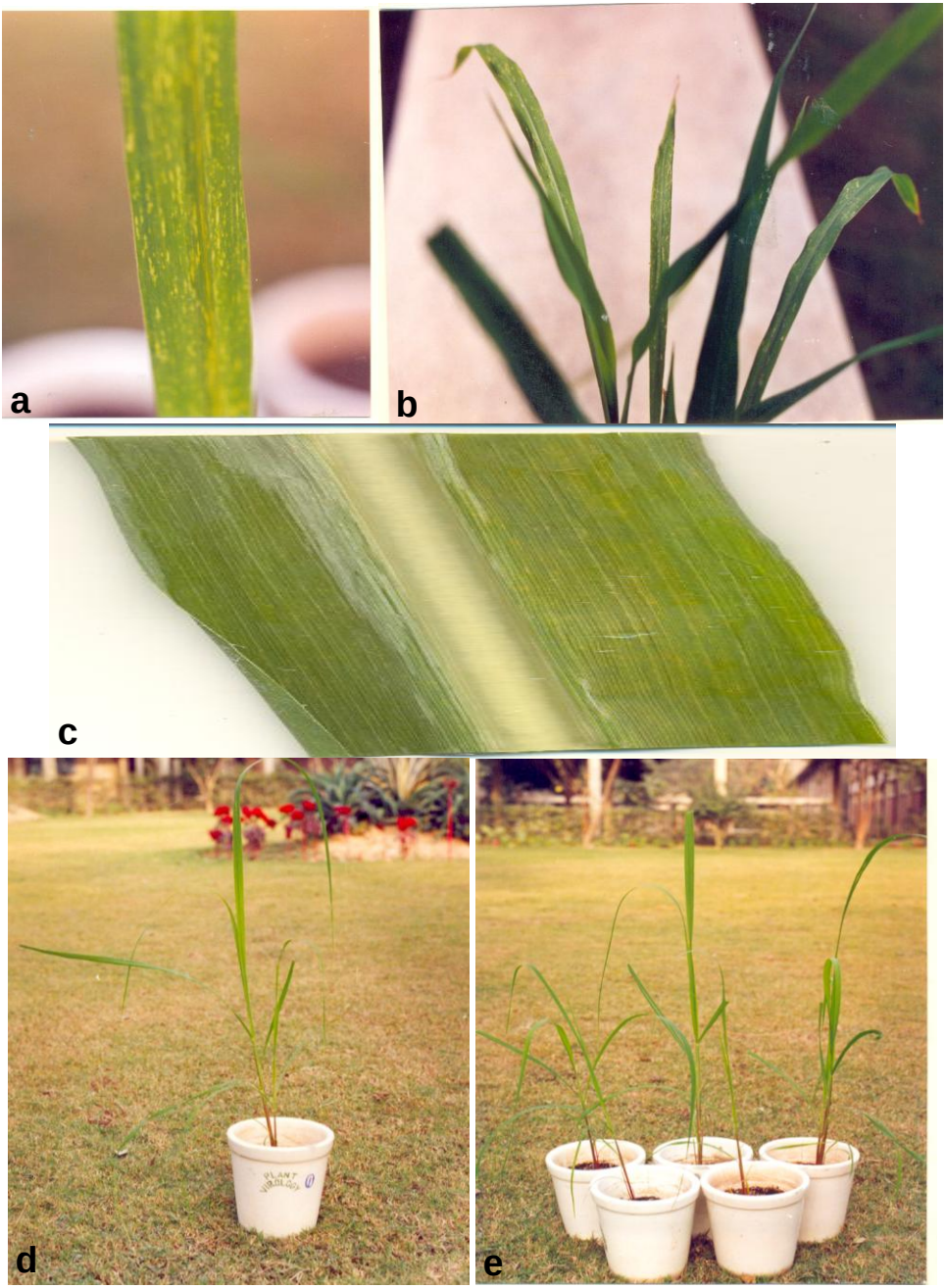

Fig. 1. a) SCMV infected sugarcane leaf (20 weeks old) streaks are visible throughout the surface of the leaf. b) Infectivity test, Development of mosaic symptoms in healthy leaves of *Sorghum bicolor* after one week of sap application from SCMV infected sugarcane leaves. c) Healthy leaf from *In vitro* rejuvenated plant. d) *In Vitro* developed mosaic virus -free plant. e) SCMV-free sugarcane plants.

Table 1. SCMV indexation of sugarcane plants regenerated through apical meristem, organogenesis and embryogenesis.

Source	No. of plants*		Infectivity Test**				Precipitin test***			
			+ve	% age	-ve	% age	+ve	% age	-ve	% age
	A**	A*								
Control ****	50	(75)	50	100.0	00	00.00	50	100.0	00	00.00
Apical Meristem	46	(63)	03	06.52	43	93.70	03	06.52	43	93.70
Organogenesis	54	(106)	21	38.88	33	61.11	21	38.88	33	61.11
Embryogenesis	66	(126)	28	42.42	38	57.57	28	42.42	38	57.57

*Selected randomly

**Based on mosaic symptoms on indicator plants (*Sorghum bicolor)*

***Based on SCMV anti-serum precipitation reaction

****Severely infected young leaves of sugarcane plants (6 weeks old). Used for the extraction of inoculum/sample.

A*In parenthesis, the total no. of plants are given

A**Randomly selected plants out of total

Table 2. SCMV indexation by Electron Microscopy of sugarcane plants regenerated through shoot meristem, organogenesis and embryogenesis.

Source	No. of plants	Plants with SCMV particles	SCMV free plants	
			Total	% age
Control****	2	2	0	00.00
Apical Meristem	5	0	5	100
Organogenesis	5	2	3	40
Embryogenesis	5	3	2	20

Discussion

Amongst the viral diseases of sugarcane, mosaic is the most important virus disease. It is believed that sugarcane mosaic virus has been distributed to all the sugarcane growing countries of the world to an extent that it is almost difficult to get single healthy sugarcane in the field (Mandahar, 1987; Pandey, 1989**).** Sugarcane yellow leaf virus (SCYLV) is distributed worldwide and has been shown to be the cause of the disease sugarcane yellow leaf syndrome (YLS) (Lehrer *et al*., 2007). Yield losses due to SCMV were reported from almost 39-46% (Mandehar, 1987; Hema *et al*., 1997). The conventional methods to overcome the viral problem are already exhausted. However, for the last two decades *In vitro* techniques have been playing significant effective role in solving the problems of plant viral infection (Quark, 1977; Short, 1991, Ahmad *et al.*, (2007). *In vitro* virus elimination technique has been successfully applied to wide range of horticulture plants and agricultural crops. Wang & Hu (1980) and Kartha (1986) reported the virus elimination through apical meristem from food crops, including *Brassica oleracea, Pisum sativum, Glycine max* and *Solanum tuberosum*.

Successful elimination of Sugarcane Mosaic Virus (SCMV) by tissue culture methods has been reported by many workers (Dean, 1982; Irvine & Benda, 1987). Peros *et al.*, 1990 published Maize Streak Virus (MSV) elimination by using bud and leaf tissues of cultured sugarcane. Chatenet *et al*., (2001) reported that sugarcane varieties from various origins were grown *In vitro* by apical bud culture and apical meristem culture and the latter proved to be the most effective method for producing SCYLV-free plant**s.** Present study also revealed the successful regeneration of virus-free plants through shoot apical meristem, organogenesis and embryogenesis. A comparative account of virus elimination potential of all the sources was determined and shoot apical meristem exhibited highest potential.

Virus indexation was carried out by a combination of infectivity tests, precipitin tests and electron microscopy. All the tests used for virus indexation are highly sensitive. It is believed that infectivity test or sap transmission of virus is one hundred times more sensitive than serological tests and electron microscopy (Barboza *et al*., 2007; Wang & Hu, 1980). It is still prevalent and widely reported method in plant virology (McDaniel & Gordon, 1985; Mandehar, 1987). For Sugarcane Mosaic Virus Indexation, most of the authors used infectivity tests and commonly used *Sorghum bicolor* and other gramineae members as indicator plants (Koike & Gillespie, 1976; Dean, 1982; Lockhart *et al*., 1992).

Among the various serology tests, Precipitin test was also used in the present study. This test is considered as relatively sensitive and extensively in use for virus detection (Kartha, 1986; Mandehar, 1987; Rao *et al*., 2001).

Present study further revealed the effectiveness of meristem size in virus elimination. Virus free plants were successfully regenerated from 0.5-5 mm meristem sizes. Many workers also reported the similar findings for eradication of viruses. (Parmessur *et al*., 2002).

Efficiency of apical meristem in regenerating virus-free stock was excellent. On the other hand, plants regenerated from organogenesis and embryogenesis exhibited relatively lower frequency of virus elimination.

The phenomenon of virus elimination through apical meristem is based on the fact that apical meristems of infected plants are generally either free or carrying very low titer of the virus (Kartha, 1986; Parmessur *et al*., 2002) The reason proposed for absence of virus are lack of vascular system, high metabolic activity of meristimatic cells, higher endogenous and exogenous level of hormones in cultured meristem cause inhibition to viral multiplication (Kartha, 1986).

Reference

Ahmad Y., L. Costet, J.H. Daugrois, S. Nibouche, P. Letourmy, J.C. Girard and P. Rott. 2007. Variation in infection capacity and in virulence exists between genotypes of Sugarcane yellow leaf virus. *Plant Disease*, 91(3): 253-259.

Ali, A., S. Naz and J. Iqbal. 2007. Effect of different explants and media composition for efficient somatic embryogenesis in sugarcane (*Sacchraum officinarum*). *Pak .J. Bot.*, 39(6): 1961-1971.

Ali, A., S. Naz, F.A. Siddiqui and J. Iqbal. 2008. Rapid multiplication of sugarcane (*Sacchraum officinarum*) through callogenesis and organogenesis. *Pak .J. Bot.*, 40(1): 123-138.

Baker, R. and H. Kinnman. 1973. Elimination of pathogens from shoot tip cultures. In: *Tissue Culture: Methods and Applications*, (Eds.): Kruse, Jr. and M.K. Patterson, Jr. pp. 735-739, Academic Press, New York.

Barboza, A.A, L. Silva, H.M. Jr, E.R. Souto, C.M. Silva, F.S. Marcuz and R.A. Vieira. 2007. Detection of Sugarcane mosaic virus in Parana state and *virus* elimination by tissue culture. *Fitopatologia Brasileira*., 32(4): 345-348.

Chatenet, M., C. Delage, M. Ripolles, M. Irey, B.E.L Lockhart and P. Rott. 2001. Detection of Sugarcane yellow leaf virus in quarantine and production of virus-free sugarcane by apical meristem culture. *Plant Disease*, 85(11): 1177-1180**.**

Dean, J.L. 1982. Failure of sugarcane mosaic virus to survive in cultured sugarcane tissue. *Plant Disease*, 66(11): 1060-1061.

Heinz, D.J and G.W.P. Mee. 1969. Plant differentiation from callus tissues of *Saccharum* species. *Crop Sci*.,9: 346-348

Hema, M., K. Sreenivzsvlu, G. Gopinath, G. Kiranmai and Satyanarayana. 1997. Partial characterization of a potyvirus causing mosaic diseases of sugar cane I Andhra Pradesh. *Indian J .Virology*, 13: 125-12.

Holling, M. and O.M. Stone. 1960. Investigation of Car nation viruses. I. Carnation mottle. *Ann. Appl. Biol.,* 53: 103-118.

Imam, P. 2001. *Sugar Industry: Problems, Prospects*. 'Daily Dawn" Economics and Business Review, February 4-10, 2002.

Irvine, J.E. and G.T.A. Benda. 1987. Transmission of Sugarcane disease in plant derived by rapid regeneration from diseased leaf tissue. *Sugarcane,* 6: 14-16.

Kartha, K.K 1986. Elimination of viruses in the presence of Antivirus chemicals production and inducing disease free plants. *Phytopathology,* 65: 219-238.

Koike, H. and A.G. Jr. Gillespie. 1976. Strain M. A new strain of Sugarcane mosaic virus. *Plant Disease Reporter,* 60(1): 50-54.

Lehrer, A.T., S. Schenck, S.L. Yan and E. Komor. 2007. Movement of aphid-transmitted Sugarcane yellow leaf virus (ScYLV) within and between sugarcane plants. *Plant Pathology*, 56(4): 711-717.

Leu, L.S. 1972. Freeing sugarcane from mosaic virus by apical meristem culture and tissue culture. *Report Taiwan Sugar Expt. Stn.*, 57: 57-63.

Liu, M.C. 1983. Sugarcane In: *Handbook of plant cell culture* 2. (Eds.): W.R. Sharp, D.A. Evans, P.V. Ammirato and Y. Yamada. Crop Science. MacMillan Publ. Co., New York, pp. 572-605.

Lockhart, B.E.L., L.J.C. Autrey and J.C. Constock. 1922. Partial purification and serology of sugarcane mild mosaic virus, A mealybug-transmitted Clostrolike Virus, *Phytopathology,* 82: 691-695.

Mandehar, C.L. 1987. Sugarcane Mosaic Disease. In: *Introduction to Plant Viruses*. (Ed.): C.L. Mandahar. pp. 524-525. Chand and Co., New Dehli.

McDaniel, L.L. and D.T. Gordon. 1985. Identification of a new strain of maize dwarf viruses. *Pl. Dis.,* 69: 602-607.

Mori, K. 1971. Production of virus free plants by means of meristem culture. *Japan Agric. Res. Quart*., 6: 1-7.

Murashige, T. 1980. Plant growth substances in commercial uses of tissue culture. In: Plant Growth Substances 1979. (Ed.): F. Skoog. Springer-Verlag, Berlin, pp. 426-434.

Murashige, T. and F. Skoog. 1962 A revised medium for rapid growth and bioassays with Tobacco Tissue Culture. *Physoil. Plant.*, 15: 473-496.

Naz, S., A. Ali and A. Siddique. 2008. Somatic embryogenesis and plantlet formation in different varieties of sugarcane (*Sacchraum officinarum*) HSF-243 and HSF-245. *Sarhad J. Agric*. 24(4): 593-598.

Panday, B.P. 1989. Diseases of Cereals. In: *Pathogen and Plant Diseases*. (Ed.): B.P. Panday. pp. 532.

Parmessur, Y., S. Aljanabi, S. Saumtally and A. Dookun-Saumtally. 2002. Sugarcane yellow leaf virus and sugarcane yellow phytoplasma: Elimination by tissue culture. *Plant Pathology,* 51: 561-566.

Peros, J.P., E. Bonnel and B. Reynaud. 1990. *In vitro* culture of Sugarcane infected with maize streak virus (MSV). *Plant Cell, Tissue and Organ Culture,* 23: 145-149.

Quark, F. 1977. Meristem culture and virus free plants. In: *Applied and Fundamental aspects of Plant Cell, Tissue and Organ Culture*. Springer-Verlag, New York, pp. 598-615.

Raja, N. and H. Abbas. 2006. Sugarcane Overview, Http://www.pakistan/com/English/allabout/crop/sugarcane. shtml cited on August 26, 2006.

Rao, G.P., R.E. Ford, M .Tosic and D.S. Teakle. 2001. *Sugarcane pathology*. Vol. II, *Virus and Phytoplsma diseases*. Oxford & IBH Publishing Co. Pvt. Ltd. New Delhi.

Rassaby, L., J.C. Girard, P. Letourmy, J. Chaume, M.S. Irey, B.E.L. Lockhart, H. Kodja and P. Rott. 2003. Impact of Sugarcane yellow leaf virus on sugarcane yield and juice quality in Reunion Island. *European Journal of Plant Pathology,* 109(5): 459-466.

Schenck, S. and A. Lehrer. 2000. Factors affecting the transmission and spread of sugarcane yellow leaf virus. *Plant Dis*., 84: 1085-1088.

Short, K.C. 1991. The physiology of cultured plantlets and methods for facilitating their transfer to field conditions. Conservation of plant genetic resources through *In vitro* methods. *ISBN*, pp. 967-991.

Singh, K. 1971.Virus diseases of sugarcane and the seed programme. *Adv. Agr*., 1: 9-87.

Slykhuis, J.T. 1976. Virus and virus like diseases of cereals crops. *Annual Review of Phytopathology*, 14: 189-210.

Sreenivasulu, P., B.C. Raju and M.V. Nayudu. 1989. Carbohydrate metabolism in mosaic virus infected sugarcane. *India Bot. Report*, 4: 129-133.

Wang, P.J. and C.Y. Hu. 1980. Regeneration of virus-free plants through *In vitro* culture. In: *Advances in biochemical Engineering*, (Ed.): A. Fiechter, pp. 61-99, Springer-Verlag, Berlin, NY.

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